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Generation of Thiocillin Variants by Prepeptide Gene Replacement and in Vivo Processing by *Bacillus cereus*

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The thiazolyl peptide antibiotics constitute a family of >80 members with the common characteristics of a central pyridine/piperidine ring typically decorated by three thiazole substituents and a macrocyclic peptide ring containing additional thiazoles[1] (Figure 1). Some members of this antibiotic group, such as thiostrepton and nosiheptide, have a second macrocyclic ring. The thiazolyl peptide natural products target one of two sequential steps in bacterial protein synthesis. Molecules such as GE2270 and thiomuracin bind tightly to EF-Tu and abrogate its aminoacyl-tRNA delivery function.[2a−c] In contrast, thiostrepton and the thiocillins bind directly to the 50S ribosomal subunit, interacting with both the 23S rRNA loops and the amino acid side chains of protein L11, with the effect of disrupting EF-G activity and thereby preventing tRNA translocation on the ribosome.[2d,e] While thiazolyl peptides display potent antibiotic activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA),[2f] poor aqueous solubility and pharmacokinetics have limited their clinical use. Furthermore, total syntheses of thiazolyl peptide compounds, while representing remarkable achievements, still present formidable challenges for structure−activity variations,[3a−f] limiting the production of novel compounds with improved pharmacokinetic properties.

Whether biosynthesis of the highly modified thiazolyl peptides occurs via nonribosomal or ribosomal assembly has long been debated;[4a] ribosomally encoded natural products[4b] are known to contain dehydroamino acids (the lantibiotics[4c,d]) and thiazoles (microcin B17, patellamide[4e,f]) like the thiocillins, but pyridine formation has not been seen in other ribosomal peptide scaffolds. Four recent reports have disclosed that GE2270, thiomuracin,[2b] nosiheptide,[5a] thiostrepton,[5b,c] and the thiocillins[5c,d] all arise by posttranslational modification of ribosomally generated prepeptides having 50−60 residues. The sequences that appear in the mature antibiotic scaffolds are derived from the C-terminus of these microbial prepeptides.

In the thiocillins from the producer *Bacillus cereus* ATCC 14579, at least 10 and up to 13 of the 14 C-terminal residues undergo posttranslational modification to generate a set of eight related antibiotics. On the basis of stable isotope feeding studies,[6a,b] the 10 core transformations are thought to involve dehydrations of Ser1[7] and Ser10 on the way to pyridine ring formation, dehydration of Thr4 and Thr13 to dehydrobutyrine residues, and cyclizations of Cys2, 5, 7, 9, 11, and 12 to six thiazoles. Three additional posttranslational modifications appear to occur stochastically: hydroxylation at Val6, O-methylation at Thr8, and/or ketone/alcohol interconversion of the C-terminal residue arising from decarboxylation of Thr14, giving rise to $2 \times 2 \times 2 = 8$ possible thiocillins. We note that four of the eight thiocillins produced abundantly by *B. cereus* display similar efficacies against *Bacillus subtilis* and two MRSA strains, with minimum inhibitory concentrations (MICs) of 0.2 to 0.9 μg/mL and <0.03 to 0.1 μg/mL, respectively [see the Supporting Information (SI)].

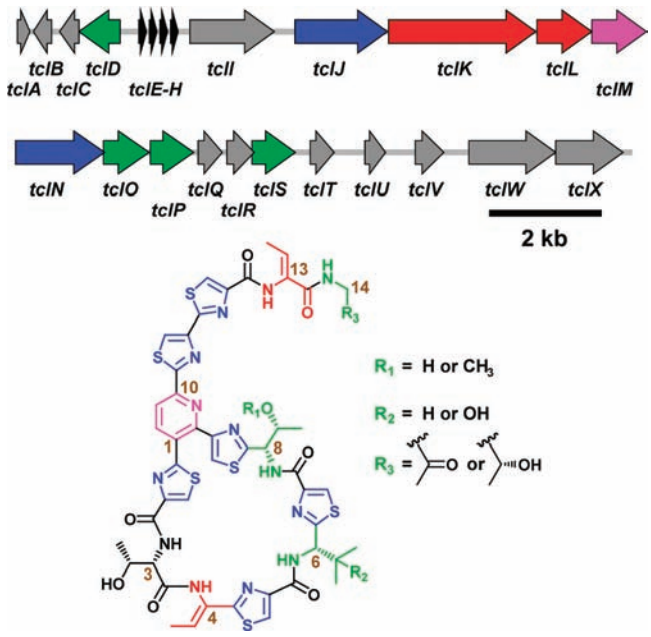

Figure 1. Annotated gene cluster responsible for the production of the thiocillins. The structure of thiocillin depicts the three positions of stochastic modification (R_1, R_2, R_3) in green.

With the recent discovery of the thiocillin gene cluster from *B. cereus*,[5d] we set out to genetically manipulate the biosynthetic machinery by site-directed mutagenesis to make modifications to the thiocillins, perhaps improving their pharmacokinetic properties without the need for new synthetic strategies. The thiocillin gene cluster contains four contiguous identical copies of a gene encoding a purported 52-residue precursor peptide (*tclE-H*), which is thought to be posttranslationally modified to yield the mature antibiotic scaffold. To confirm that the tandem genes *tclE-H* are responsible for generating the thiocillin prepeptide, we generated a *B. cereus tclE-H* knockout strain (*tcl*Δ*E-H*) by homologous recombination with a plasmid containing sequence homology to the *tcl* gene cluster but lacking *tclE-H* (see the SI). Cultures of wild-type (WT) *B. cereus* ATCC 14579 and *tcl*Δ*E-H* were extracted for compound and analyzed by reversed-phase HPLC. WT *B. cereus* extracts contained thiocillins, as observed by UV absorption at 350 nm and LC−MS (Figure 2). In contrast, extracts of *tcl*Δ*E-H* failed to yield any of the eight thiocillin compounds. To rescue production of the thiocillins and confirm tclE as the prepeptide responsible for thiocillin production, we inserted a single plasmid-based copy of *tclE* into the chromosome of *tcl*Δ*E-H* by Campbell integration.

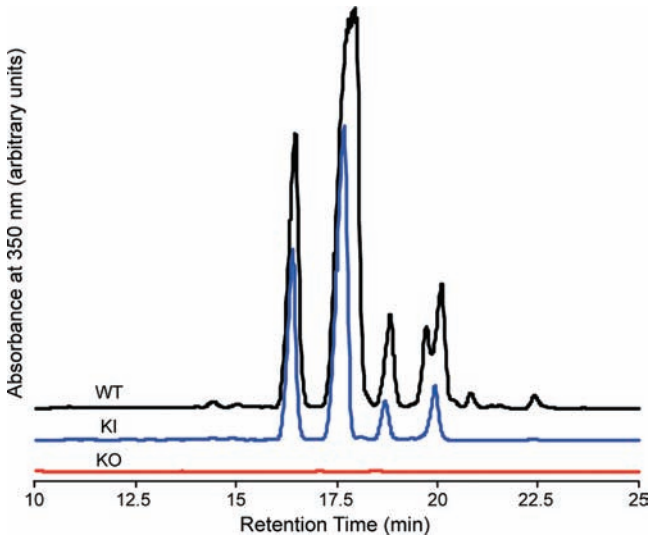

Figure 2. HPLC traces of thiocillin extractions from *B. cereus* ATCC 14579 (WT), tclE-H knockout *tcl*Δ*E-H* (KO), and *tclE KI* (KI).

Cultures of the knock-in strain (*tclE KI*) were extracted and analyzed by reversed-phase HPLC and LC−MS, which revealed that *tclE KI* rescued production of thiocillins to near-WT levels (Figure 2).

The ability to rescue thiocillin production with a *single* plasmid-based copy of *tclE* enables the mutasynthesis of novel thiocillin compounds in *B. cereus* by use of variant *tclE* genes to initiate structure−activity relationship studies of the thiazolyl peptide antibiotics. We initially focused on residues 3, 4, 6, 8, and 13, which are not involved in setting the trithiazolylpyridine core in the mature scaffold, reasoning that amino acid substitutions at these positions probe the promiscuity of the thiocillin tailoring enzymes while minimizing disruption to the core framework. In all, 14 single amino acid substitutions were made in the thiocillin prepeptide tclE at these five positions, and 12 resulted in production of one or more thiocillin variants (Table 1 and SI Table 6.1 in the SI). The combination of possible posttranslational modifications at positions 6, 8, and/or 14 gave rise to 65 thiocillin molecular variants detected by LC and high-resolution MS (HRMS) (SI Table 6.1).

In a first set of site-directed mutants of the *tclE* gene, amino acids T3, V6, T8, and T13 were substituted with alanine and T4 with valine, all of which are relatively conservative changes, minimizing the existing side chains to methyl or isopropyl groups. The *tclE* mutant plasmids were each transformed into *B. cereus* *tcl*Δ*E-H*. HPLC analysis of extracts from 0.5 L cultures confirmed the presence of a number of thiocillins. The T3A variant produced six of the eight expected thiocillins, while four thiocillins were observed in T8A, where A8 cannot be methylated. T4V generated four of the eight expected compounds plus two additional derivatives with masses corresponding to the addition of two hydroxyl groups, suggesting that tclD, the V6 hydroxylase, is also able to hydroxylate a valine at position 4. Further evidence of the promiscuity of tclD is the identification of forms with hydroxylated Cβ of the alanine side chain among the six compounds isolated from the V6A variant.

To determine antimicrobial activity, the extracts from each compound were subjected to normal-phase chromatography on silica gel, and fractions containing compounds absorbing at 350 nm were collected. Because the individual thiocillin derivatives produced by WT *B. cereus* inhibited bacterial growth with similar MICs, the tclE variants from a given mutant were pooled for

Table 1. Summary of Thiocillin Variants Produced in This Study and Their Antibiotic Efficacies against *B. subtilis* and Methicillin-Resistant *S. aureus*

	mutant	compounds observed[b]	additional modifications	MIC (μg/mL)[a] 168[c]	COL[d]	MW2[e]
1	T3K	4	N-succinylation	>8	>8	>8
2	T3D	4	−	>8	>8	>8
3	T3A	6	−	>100	>100	>100
4	T4K	4	−	n.t.[f]	n.t.	n.t.
5	T4V	6	−	>100	>100	>100
6	V6D	N/A	−	n.t.	n.t.	n.t.
7	V6K	N/A	−	n.t.	n.t.	n.t.
8	V6A	6	*Ala*-hydroxylation	0.13	0.06	0.06
9	T8C	7	S-methylation, Cys-cyclization	0.3	0.15	0.08
10	T8A	4	−	1	0.25	0.25
11	T8K	6	N-succinylation	>4	>4	>4
12	T13A	7	−	0.13	0.06	0.06
13	WT[g]	6	−	0.5	0.06	0.13

[a] Minimum inhibitory concentrations determined by overnight culture in 96-well plate format. [b] For details, see the SI. [c] *B. subtilus* 168. [d] Methicillin-resistant *S. aureus* COL. [e] Methicillin-resistant *S. aureus* MW2. [f] n.t. = not tested. [g] WT = wild type.

antibiotic activity assays by disk diffusion on LB plates containing *B. subtilis* strain 168. The V6A, T8A, and T13A variants maintained levels of antibiotic activity similar to that of the WT thiocillin set (Table 1). In contrast, the T3A and T4V derivatives failed to inhibit growth at amounts up to 8 μg, suggesting that these particular variations disrupt the binding or positioning of the compounds at the L11/23S rRNA binding interface on the large ribosomal subunit. To quantify the antibiotic activity, MICs were determined in serial-dilution liquid culture assays with both *B. subtilis* and *S. aureus*. The V6A and T13A variants showed 2−4-fold improved activity against *B. subtilis* and MRSA strain COL and were equally active against MRSA strain MW2. In contrast, T8A showed slightly decreased antibiotic activity.

To introduce charge, T3D and V6D were then generated to determine whether the side-chain carboxylate anions, which could potentially improve the solubility of the thiocillin scaffold, can be accepted in these positions by the nine posttranslational-modification open reading frames *tclDJKLMNOPS*. Cultures of the V6D mutant failed to produce any thiocillin compounds, as determined by LC and HRMS. In contrast, T3D produced four of the eight expected compounds in sufficient quantity to be purified; however, at amounts up to 8 μg, the T3D variants failed to inhibit growth of *B. subtilis*. Positions T3 and V6 were also substituted with lysine. No thiocillin compounds were identified from extracts of the V6K mutant. Although production levels were significantly reduced from those of WT thiocillin, requiring growth in a 5 L fermenter, the T3K variant produced two thiocillin compounds. As with T3D, T3K was inactive against *B. subtilis* in disk diffusion assays containing up to 8 μg of compound.

LC−MS analysis of extracts of T3K identified multiple compounds with additional mass increases of 100.106 Da, suggesting $C_4H_4O_3$ as the added functional group. MS/MS analysis confirmed the extra mass in all of the fragments containing lysine 3, and N-succinylation was confirmed by MS/MS and NMR analysis (see the SI). N-Succinylation of T3K and T8K, presumably by succinyl-CoA, may be a self-protection strategy of the producer organism. N-Succinylated T3K derivatives with the extended carboxylate side chain were as inactive

as the unmodified T3K thiocillin scaffold in disk diffusion assays. The corresponding T4K and T8K mutants in the *tclE* gene were next explored. T8K generated all four of the expected compounds (no 8 O-methylation) as well as three N-succinylated derivatives. T4K produced four compounds, all of which were methylated at position T8. Interestingly, not a single N-succinylated derivative of T4K was observed. Production of T4K was insufficient for antibiotic activity analysis, and T8K failed to inhibit the growth of either *B. subtilis* or MRSA.

As a third test of the thiocillin biosynthetic processing machinery to accept tclE prepeptide modifications, we introduced a cysteine substitution as variant T8C. In the maturation of WT thiocillin, all six cysteine residues in the 14-amino acid C-terminus of tclE are converted to thiazoles. The addition of a seventh cysteine could give one of the following distinct outcomes: (1) complete conversion to a seventh thiazole, as for the six native cysteines, via an intermediate thiazoline; (2) S-methylation of C8 in analogy to O-methylation of T8 in the native tclE; or (3) no modification. Extracts of the T8C variant contained near-WT levels of compounds, and LC−MS analysis identified seven thiocillins. No thiazole at residue 8 was detected, but three of the four expected thiazoline derivatives were identified by MS/MS; the *S*-methyl Cys8 forms predominated. Disk diffusion assays confirmed the antibiotic activity of T8C compounds against *B. subtilis*, and MIC values against *B. subtilis*, MRSA COL, and MRSA MW2 indicated retention of almost full antibiotic efficacy.

The results presented herein begin to decipher the functional requirements for processing of the 52-residue tclE prepeptide to the mature thiocillin scaffold and the stochastic variants at side chains 6, 8, and 14. This is also a beginning for mapping of the antibiotic activity of thiocillin variants. In all, 65 novel thiazolyl peptide compounds were generated. While conservative variants at positions 6, 8, and 13 maintained considerable or equivalent antibiotic activity, those at positions 3 and 4 as well as more drastic charge-insertion mutants were completely inactive over the concentration ranges tested. These results correlate well with the position of micrococcin modeled into the complex with the 50S ribosome by Harms and colleagues.[2e] Threonine-3 and dehydrobutyrine-4 appear in close proximity to ribosomal protein L11; disrupting these contacts could result in perturbation of micrococcin binding to the ribosome and loss of antibiotic activity.

The ability to express *tclE* gene mutants in a *B. cereus* strain whose four tandem endogenous copies of *tclE-H* have been deleted sets the stage for more extensive structure−activity evaluations. These include alterations that still allow processing to the mature trithiazolylpyridine core in this highly morphed ribosomal peptide antibiotic framework (e.g., the requirement for three thiazoles surrounding the pyridine core and the size and flexibility of the macrocyclic ring connecting thiazoles 2 and 9). Fermentation of the variant thiocillins will also allow evaluation of the subset of scaffolds that retain antibiotic activity and show improvements in such parameters as aqueous solubility.

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Supporting Information Available: Supporting figures and tables, experimental procedures, and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Thiocillin numbering: Ser1−Thr14, where Ser1 is Ser39 of the prepeptide.

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